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Molecular cloning and characterization of a novel orphan receptor (P_{2P}) expressed in human pancreas that shows high structural homology to the P_{2U} purinoceptor

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Nico J. Stam*, Jan Klomp, Marcel van de Heuvel, Wiebe Olijve

N.V. Organon, Department of Biotechnology and Biochemistry, P.O. Box 20, 5340 BH Oss, The Netherlands

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Abstract Here we report the cloning of a gene encoding a new member of the superfamily of G protein-coupled receptors. The gene encodes a protein of 365 amino acids closely resembling two recently cloned nucleotide binding receptors, called P_{2U} and P_{2Y} purinoceptors (71% and 49% sequence identity within the transmembrane domains, respectively). Our studies show that this new putative purinoceptor (designated P_{2P}) is encoded by an intronless single copy gene that is exclusively expressed in pancreas, in contrast to the P_{2U} and the P_{2Y} purinoceptors which are widely distributed throughout the periphery. The identification of a pancreas-specific human putative P₂ purinoceptor makes it attractive to speculate that the reported actions of ADP/ATP analogues in pancreas on insulin secretion are mediated through this receptor.

Key words: G-protein-coupled receptors; ATP; ADP; Purinoceptor; P_{2P} subtype; Human pancreas

1. Introduction

Nucleotides such as ATP, ADP, and UTP act as intercellular neurotransmitters and exert a broad range of physiological responses in both the central and peripheral nervous system, such as insulin secretion, vasodilatation, platelet aggregation, wound healing, and transepithelial ion transport [1-6]. These effects are mediated via activation of extracellular membrane receptors, known as P₂ purinoceptors, to distinguish them from the P₁ purinoceptors that bind adenosine (reviewed in [7,8]). The existence of multiple peripheral purinoceptors may help account for some of the complex physiological responses thought to be brought about by extracellular nucleotides. At least five P₂ purinoceptor subtypes (P_{2X}, P_{2Y}, P_{2U}, P_{2T}, P_{2Z}) have been identified on the basis of pharmacological criteria, the most important of which is their rank order of potency for a number of reference compounds [8]. The classification of P₂ purinoceptor subtypes, however, is seriously hampered by the lack of selective (ant)agonists as well as the chemical instability of some of the nucleotide ligands [8].

The recent cloning of three members of the P₂ purinoceptor family has considerably improved our understanding of the structure and mechanism of activation of purinoceptors [7,9-14]. Moreover, the elucidation of the protein structure has added an additional criterion to allow adequate receptor classification. Whereas the predicted amino acid sequence of the P_{2X} purinoceptor shows resemblance to members of the ligand-gated ion channels, the cloned P_{2U} and P_{2Y} purinoceptor showed the presence of a putative seven-transmembrane struc-

ture, a hallmark of receptors that couple to guanine nucleotide binding (G) proteins [18]. In this study we have exploited the structural resemblance among receptors that couple to G-proteins to clone a novel member of the P₂ purinoceptor family. Using polymerase chain amplification of genomic DNA with degenerate primers corresponding to conserved transmembrane domains of previously cloned serotonin receptors [6,17], we identified a novel P₂ purinoceptor. Here we report the genomic cloning and characterization of a gene encoding a new P₂ purinoceptor subtype that is exclusively expressed in pancreas. The identification of a pancreas-specific P₂ purinoceptor is especially of interest in view of the reported actions of ADP analogues on insulin secretion via a pharmacologically defined P₂-like purinoceptor in pancreas [5,6,21,22].

2. Materials and methods

2.1. General methods

All general recombinant DNA procedures were performed as described by Sambrook et al. [15]. Restriction endonucleases were purchased from Pharmacia. Radiolabelled nucleotides were from DuPont (NEN).

2.2. PCR amplification

Total human genomic placenta DNA, purchased from Clontech Laboratories (Palo Alto, CA, USA), was used as a template for polymerase chain (PCR) amplification with a set of degenerate oligonucleotide primers corresponding to conserved sequences of the third and sixth transmembrane domains of known G-protein-coupled serotonin (5-HT) receptors. One of the primers contained inosine as base substitute to avoid too much sequence degeneracy following the original paper of Libert et al. [16]. The sequences of the forward and reverse primers were, respectively:

TM3F 5'-CTGTGCGT(CG)ATCAGC(CA)T(CG)GAC(CA)-
G(GC)TA-3';
TMR6R 5'-GAAAAAIGGGCACCA(GC)ATAT(CA)(AG)(AG)-
AA-3'.

The conditions of the PCR were as follows: after denaturation at 94°C for 4 min, 35 cycles (94°C, 55°C, 30 s; 72°C, 1 min) and an additional extension at 72°C for 4 min were carried out using a Perkin-Elmer 9600 thermal cycler. Amplification reactions contained, in a total volume of 100 µl, 0.3 µg human genomic DNA, 2.5 units AmpliTaq polymerase (Perkin Elmer), 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 3% DMSO, and 0.2 mM of each dATP, dCTP, dGTP, and dTTP. The amplified DNA fragments (400-700 base pairs long) were analyzed in a 1.5% agarose gel. Individual bands were excised, extracted with phenol, precipitated with ethanol, and 5% was used as template in a second PCR. The amplified DNA was analyzed on agarose gel and individual bands were subcloned into the PCRII vector (Invitrogen) for sequencing. Out of six bands analyzed, one was found to have an unreported sequence of approximately 410 bp insert that showed homology with G-protein-coupled receptors. This fragment was subsequently used to probe a human genomic DNA library as described elsewhere [17].

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*Corresponding author. Fax: (31) (412) 6625533.

2.3. Genomic DNA library screening and DNA sequencing

A genomic DNA library prepared from placenta DNA partially digested with *Sau3A* restriction enzyme and subcloned into EMBL3 sp6/t7 phage vector was purchased from Clontech Laboratories (Palo Alto, CA, USA). The library was plated and duplicate plaque lift filters were prepared according to standard protocols [14]. Approximately 2 million recombinants were probed with the 410-bp PCR-generated fragment that was radiolabeled with [³²P]dCTP by the random priming method. Plaques showing positive hybridization to both filters were isolated and purified through two more rounds of screening. Hybridization conditions and washing conditions were essentially the same as described previously [17].

2.4. Oligonucleotides, probes, labelling and hybridization analysis

Oligonucleotides used for PCR and sequencing were synthesized on an Applied Biosystems 392 DNA synthesizer. They were eluted from the column with a solution of 32% (v/v) ammonia for 60 min. The eluate was heated overnight at 55°C and oligodeoxynucleotides were desalted by ethanol precipitation. The following oligonucleotides were used for PCR amplification reactions as well as for sequencing:

p3	(5'-TGCTATGGACTCATGCTCGTCGCC-3' sense)
p5	(5'-TCGGCCTGAAGAGTTTGACCACTA-3' sense)
p6	(5'-GGCATCTGCCACCACTTCGG-3' sense)
p7	(5'-CCGAATTGGGTGGCAGATGCC-3' antisense)
p4	(5'-CAGCTATGGTGCGGAGAGAGCGGA-3' antisense)
p8	(5'-GTCCGAGGCGGAAGATGAAGAG-3' antisense)
p9	(5'-GCCATGGCCAGTACAGAGTC-3' antisense)

Probes used for genomic library screening (probe 1) as well as for Southern blot and Northern blot analysis (probe 2) were generated by PCR amplification using pOR3 DNA as a template with specific sets of primers flanking the indicated region. PCR conditions were as described [16]. Probe 1 corresponds to nucleotides 372–782 and was amplified using the primer set P1/P2. Probe 2 corresponds to nucleotide –3–196 and was amplified with primer set p8/p9. The amplified fragments were purified by agarose gel electrophoresis, labelled with [α -³²P]dCTP by the random priming method [15].

2.5. mRNA and genomic DNA analysis

Human genomic DNA blots were purchased from Clontech Laboratories (Palo Alto, CA, USA). These blots are positively charged nylon membranes to which restriction enzyme digested genomic DNAs have been transferred by Southern blotting after being separated in a 0.8% agarose gel. Each lane contains 8 μ g of genomic human kidney DNA. Prehybridization, hybridization conditions and washing conditions were performed at high stringency following the suppliers protocol. The filters were prehybridized for 4 h at 65°C in a solution containing 5 \times SSPE, 10 \times Denhardt's solution, 100 μ g/ml freshly denatured, sheared salmon sperm DNA. SSPE (20 \times) = 3 M NaCl, 0.3 M Na₂citrate-2H₂O, pH 7.0, Denhardt's solution (50 \times) = 5.0 g Ficoll (type 400, Pharmacia), 5.0 g polyvinylpyrrolidone (Sigma), 5.0 g bovine serum albumin in 500 ml. Hybridization was performed overnight at 65°C in the same buffer with probe 2. The filter was washed two times for 20 min each in solution A (2 \times SSC, 0.5% SDS) at 60°C, and three times for 15 min in solution B (0.1 \times SSC, 0.1% SDS) at 60°C.

Multiple tissue Northern blots were purchased from Clontech Laboratories. Each lane contains approximately 2 μ g of poly(A)⁺ RNA from various human tissues, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes. RNA isolation, and construction of the blots, is described in the manufacturer's protocol. Hybridization was performed overnight at 42°C in hybridization buffer (described in supplier's protocol). After hybridization, the filters were washed as indicated in the above mentioned protocol.

3. Results and discussion

To isolate unknown genes that might encode novel members of the G-protein-coupled receptor family, we used polymerase chain reaction amplification with highly degenerate oligonucleotide primers corresponding to conserved regions

in the third and sixth transmembrane domains (TMDs) of previously cloned members of this family [17,18]. The PCR products were analyzed by gel electrophoresis in a 1.5% agarose gel and ethidium bromide staining. Amplified DNA fragments in the range of 0.4–0.6 kb were subcloned and characterized by sequence analysis. One of the clones contained a 410 bp insert of unreported sequence that encoded an amino acid sequence homologous to members of the G-protein-coupled receptors. The insert of this clone was radiolabelled and used to screen a human genomic DNA library at high stringency to isolate the complete gene. This process resulted in the isolation of a 16 kb human genomic DNA insert in EMBL lambda phage, designated pOR3. Southern blotting and restriction mapping showed the hybridizing region to be contained on a single 2.4 kb *SacI* fragment. Sequencing of this *SacI* fragment revealed an intronless open reading frame of 1095 bp corresponding to a predicted 365 amino acid residue protein with an estimated molecular mass of approximately 41 kDa. Because of its structural resemblance with P₂ purinoceptors and its selective expression in pancreas (see below), the receptor cloned is hereafter referred to as P_{2P} purinoceptor. The nucleotide sequence and the deduced amino acid sequence of the P_{2P} receptor are shown in Fig. 1. The open reading frame starts with an ATG initiator codon for translation (nucleotides 1–3) that matches the consensus sequence for initiation of translation and the presence of an in-frame stop codon upstream of this methionine codon indicates that the clone encodes the entire coding region. A termination codon (nucleotides 1093–1095) occurs in-frame after the codon specifying a leucine. The deduced amino acid sequence shares characteristic structural features with members of the G-protein-coupled receptor family [18].

Hydrophobicity analysis of the predicted amino acid sequence of P_{2P} revealed the presence of seven stretches of hydrophobic residues of about 25 amino acids, a feature shared by all members of the G-protein-coupled receptor family. The P_{2P} purinoceptor also contains several other structural features that are apparent in all members of the G-protein-coupled receptor family, including: (1) two cysteine residues (positions 109 and 185) in the first and second extracellular loop that are presumed to form a disulfide bond that potentially form a disulfide bridge that stabilizes the receptor conformation; four proline residues within transmembrane regions 4–7 (Pro¹⁶⁹, Pro²¹², Pro²⁶⁰, and Pro³⁰³) that may play crucial roles in helix arrangements and that are presumed to play a crucial role in ligand binding and transmembrane signalling; and a conserved cysteine (Cys³²⁰) in the cytoplasmic tail distal to TM7 that may be modified by palmitoylation and that could be used to anchor the tail to the plasma membrane [18].

A comparison of the deduced amino acid sequence of P_{2P} with previously cloned G-protein-coupled receptors in the EMBL GenBank data base revealed the highest degree of sequence identity with the recently cloned P_{2U} and P_{2Y} purinoceptors (overall sequence identity: 50% and 30% respectively). Within the transmembrane domains the identity is even higher (71% with P_{2U} and 49% with P_{2Y}). A comparison of the predicted amino acid sequence of P_{2P} with the recently cloned human P_{2U} and P_{2Y} receptors is schematically shown in Fig. 2. Furthermore, the dendrogram of several G-protein-coupled receptors shows the highest homology of the P_{2P} receptor with the P_{2U} purinoceptor (Fig. 3).

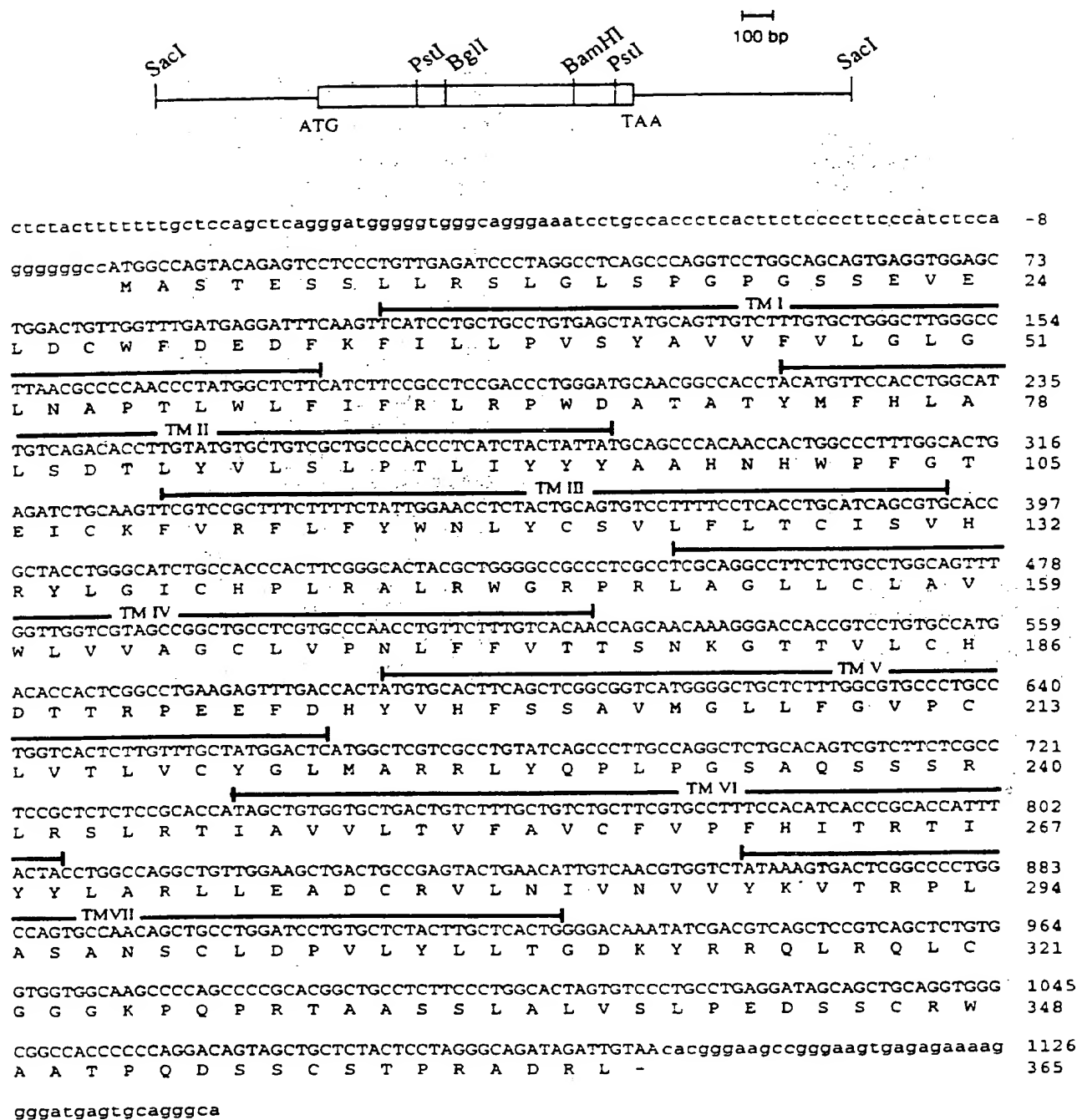


Fig. 1. Restriction map and nucleic acid sequence of clone pOR5 (P₂ purinoceptor). Top, the map depicts a portion of clone pOR5, with the 5' end of the sense strand to the left and the coding region boxed. Relevant restriction enzymes are marked. Bottom, nucleotide sequence of the human P_{2P} purinoceptor gene. The DNA sequence is numbered at the right, beginning with the translation initiation codon. The amino acid numbering is indicated below the nucleotide numbering. The positions of the putative transmembrane regions (I-VII) are depicted by lines above the nucleotide sequence. The nucleotide sequence of the human P_{2P} purinoceptor has been submitted to the Genbank/European Molecular Biology Lab database under acquisition number X96597.

Several interesting structural features that are apparent for the P_{2U} and P_{2Y} purinoceptors are also conserved in the cloned P_{2P} receptor: whilst most of the G-protein-coupled receptors contain an aspartic acid (DRY) at the interface between TMR3 and the second cytoplasmic loop [18], this aspartic acid is replaced by an histidine (HRY) in both P_{2U}, P_{2Y}, and P_{2P} purinoceptors [7]. Also worth noting is the pre-

sence of two arginines (position Arg²⁶⁵ and Arg²⁹²) as well as a histidine (His²⁶²) in transmembrane region VI and VII in the P_{2P} purinoceptor, at the same relative position as was found in the human P_{2U} and P_{2Y} purinoceptors (reviewed in [7]). Moreover, using site directed mutagenesis techniques it was recently shown that these conserved positively charged amino acids in the P_{2U} purinoceptor are involved in the binding of

the negatively charged phosphates of ATP and UTP ligands [19]. Thus, on the basis of the high degree of sequence homology with the P_{2U} and P_{2Y} purinoceptors, as well as the conservation of several key residues presumed to be involved in binding of nucleotides it is highly likely that the cloned P_{2P} receptor gene encodes a new member of the purinoceptor family.

To determine the number of genes encoding the P_{2P} receptor, human genomic DNA was digested with several different restriction enzymes, separated on 0.8% agarose, transferred to nylon filters and hybridized using a specific probe for the P_{2P} receptor (Fig. 5). Under high stringency conditions, single hybridizing bands were obtained in all lanes suggesting that P_{2P} is a singly copy gene.

Northern blot analysis was performed to investigate the expression of the P_{2P} purinoceptor in human tissues. As shown in Fig. 4, a single prominent mRNA species of approximately 2.6 kb, was detected in pancreas, despite the fact that a relatively small amount of RNA was transferred to this lane, as visualized by the actin hybridisation. No evidence for expression was found in any other tissue examined, including: heart, brain, placenta, lung, liver, skeletal muscle, kidney, spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes.

One striking feature that discriminates the P_{2P} purinoceptor from the P_{2U} and P_{2Y} purinoceptors is its tissue distribution. Whereas the P_{2U} and P_{2Y} purinoceptors are widely distributed throughout the periphery and the CNS [10-12], the P_{2P} purinoceptor is exclusively expressed in pancreas. Its unique tissue distribution is especially of interest in the light of several studies that showed profound effects of ADP nucleotides on insulin secretion [5,6,21,22]. In these studies it has been pro-

P2Y	KTEVLWPAVPGTDAAFACPGSSWGNSTVASTAAVSSSFKALTKTGQFY	52
P2U	MA-----ADLGP-----WNTINCTWGGDELYRCRF-NEDFKYV	34
P2P	MA-----STESSLL-----RSGLSPGSGSEVELDWF-DEDFKZ	36
I		
P2Y	YLPAYVYLVTIGLGNVAIWHVFHKKWPGSGISVYHFNALADFLVLT	104
P2U	LLPVSYGVVGVLCGLNAVGLYIFLCRLKTNASTTYHFLAVSDALYAASL	86
P2P	LLPVSYAVVTVLGLNAPFLWLFIFRLRPWDATATYHFLASDLYLVL	88
II		
P2Y	PALIFYYFNKTDFPGDAMKCLQRFIFHVNLY-GILFLTCISAHRYSGVYF	155
P2U	PLLVYYARGDHWFFSTVLCCLVRFLFYTNLYCSILFLTCISVHRCGLVLP	138
P2P	PLLVYYAHHNWFPGTEICKFVRFLLTYNLYCSVLFLTCISVHRYLGICHP	140
III		
P2Y	LKSLGRLLKKNIAICISVLVWLVVVVAISPLFYSGTGVRKNTITCYDTSD	207
P2U	LRSLRWGRARYARRVAGAVMVLVLAQAPVLYFVTTSEAR-GP-LTCHDTSAP	188
P2P	LRALLRWGRPRLAGLLCLAVVLVAGCLVPLNFVTTSENK-GTTLVCHDTTP	191
IV		
P2Y	EYLSRYFYSHCTTAMFCVPLVLILGCGYGLIVRALIYKDL-----DNSPLR	254
P2U	ELFSRFVAYSSVMGLLFAVPAVILVCTVLMARRL-LKPAYGTSGGLPRAX	239
P2P	EEFDHYHFSSAVMGLLFGVPCLVTLVCTGLMARRL-YOPLPGSAQSSRLR	242
V		
P2Y	RKSIYLVILVTVFAVSYPFHVMKTHNLRAR-LDFOTPAHCAFNDRVYATY	305
P2U	RKSVRTIAVVLAVTALCLFPPHVTRTLTYYSFRSLDLS-----CHTLNAINMAY	287
P2P	--SLRTIAVVLTVFAVCFVPHITRTIYYLARLLLEAD-----CRVLNIVNVVY	288
VI		
P2Y	QVTRGLASLNSCDVPIYFLAGDT-----FRRLSRAT-----RKASRRSEANLQ	350
P2U	KVTR-LASANSCLDPVLVYFLAGQLVRFARDAKPPPTGSPATPARRTLGLRR	338
P2P	KVTRPLASANSCLDPVLVYLLTGDK-----YRRLQLRLCGGKPOP-----R	329
VII		
P2Y	SKSEDMTL--NILPEFKQ-----NGDTSI	372
P2U	SDRTDMRIGDVLGSSDSRRTESTPAGS-ENTKDIRL	375
P2P	TAASSLALVS--LPDSSCRMAATPDSSCSTPRADRL	365

Fig. 2. Comparison of the pOR5 (P_{2P} purinoceptor), the human P_{2U} and P_{2Y} purinoceptor deduced amino acid sequences. The seven putative transmembrane domains (I-VII) are indicated above the amino acid sequence. Gaps have been introduced to maximize alignment. Residues that are identical in the alignment of P_{2P} , P_{2U} , and P_{2Y} purinoceptors are labelled with a '*' below the sequence. Residues labelled with a '.' below the sequence indicate conserved residues.

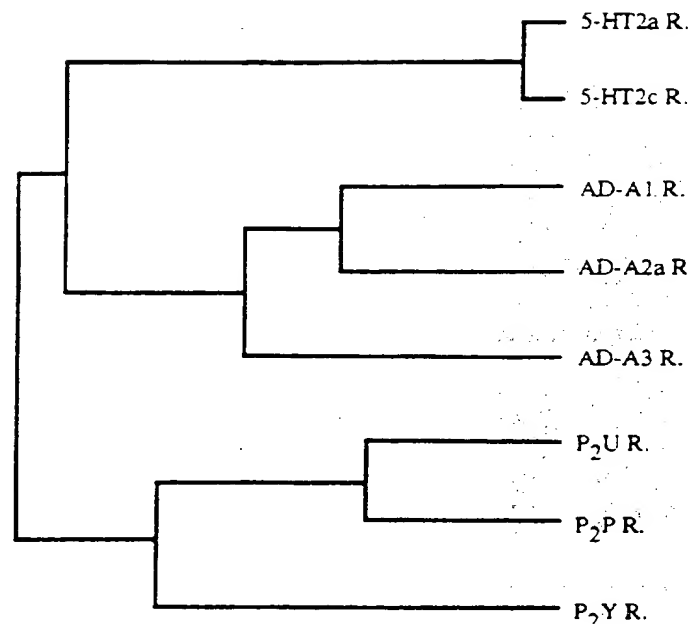


Fig. 3. Dendrogram of several G-protein-coupled receptors, achieved by the method of average linkage cluster analysis using the PC/ Gene, CLUSTAL program [20], shows the most similar sequences besides each other. The shown distances are inversely proportional to the sequence homology. AD-A1 R., AD-A2a R. and AD-A3 R. are the adenosine receptors A1, A2 and A3, respectively. P2Y and P2U are the recently cloned purinoceptors.

posed that the effects of ADP analogues on insulin secretion are mediated by a P_2 purinoceptor. The finding of a P_{2P} purinoceptor which is exclusively expressed in pancreas makes it attractive to speculate that the reported effects of ADP analogues on insulin secretion are mediated through the presently identified receptor. In view of this, it will be important to determine the precise anatomical distribution of the P_{2P} purinoceptor within the pancreas, e.g. via in situ hybridization, to see whether it is expressed in the β -cells of the pancreas. Furthermore, it will be important to determine whether the pharmacological profile of the cloned receptor is similar to that of the previously identified pancreas receptor. To this end, experiments are aimed to express the cloned gene in eukaryotic host cells, lacking endogenous P_2 purinoceptors. In case the purinoceptor described here indeed plays a role in the secretion of insulin it may be a target for new antidiabetic drugs.

4. Note added in proof

During the submission of the manuscript a clone with a similar sequence was reported by Communi et al., J. Biol. Chem. 270 (1995) 30849.

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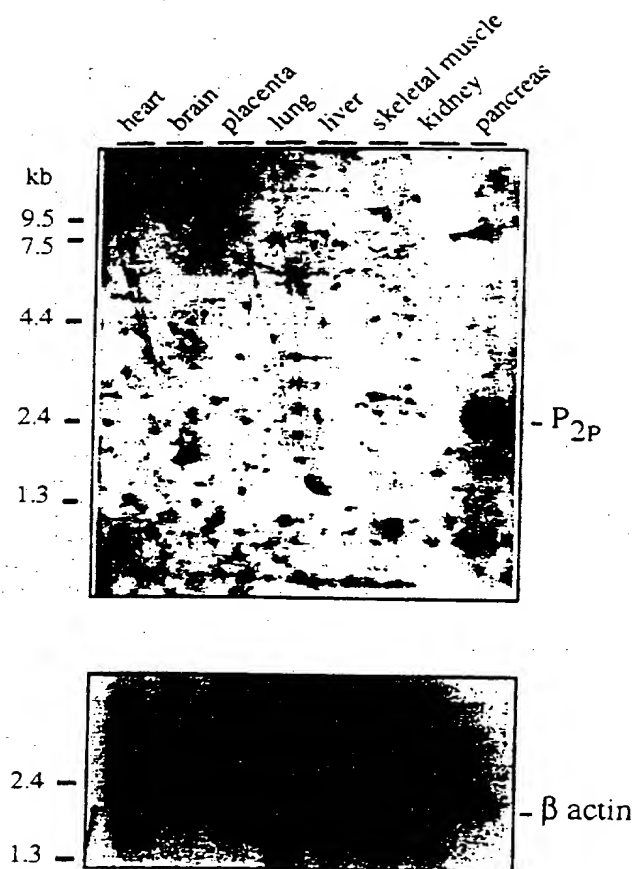


Fig. 4. Northern blot analysis of the P_2 purinoceptor in various human tissues. Each lane contains 2 μ g of poly(A)⁺ RNAs extracted from various human tissues. The blot was subsequently hybridized using two ³²P-labelled probes specific for P_2P purinoceptor and actin, respectively. RNA molecular size markers are indicated on the left.

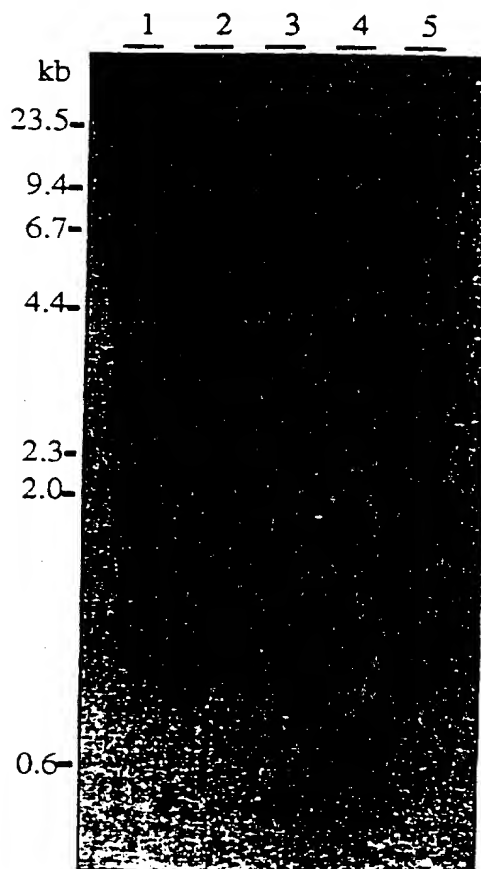


Fig. 5. Southern blot hybridization analysis of human genomic DNA. 8 μ g of human genomic DNA was digested with various enzymes (Clontech, Palo Alto), separated on 0.8% agarose gels and transferred to nylon filters. The filter was hybridized with a probe specific for pOR5 (probe 2, section 2.4). Numbers above the lane indicate the restriction enzymes used to digest the DNA: lane 1, *EcoRI*; lane 2, *HindIII*; lane 3, *BamHI*; lane 4, *PstI*; and lane 5, *BglI*. DNA size markers are indicated on the left.

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